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Structural and mutational analysis of amino acid residues involved in ATP specificity of Escherichia coli acetate kinase.

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3

4 Short title: Specificity to ATP of acetate kinase

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17

18 **Abstract**

19 Acetate kinase (AK) generally utilizes ATP as a phosphoryl donor, but AK
20 from *Entamoeba histolytica* (PPi-ehiAK) uses PPi, not ATP, and is PPi-specific.
21 The determinants of the phosphoryl donor specificity are unknown. Here, we
22 inferred 5 candidate amino acid residues associated with this specificity, based on
23 structural information. Each candidate residue in *Escherichia coli* ATP-specific
24 AK (ATP-ecoAK), which is unable to use PPi, was substituted with the respective
25 PPi-ehiAK amino acid residue. Each variant ATP-ecoAK had an increased K_m for
26 ATP, indicating that the 5 residues are the determinants for the specificity to ATP
27 in ATP-ecoAK. Moreover, Asn-337 of ATP-ecoAK was shown to be particularly
28 significant for the specificity to ATP. The 5 residues are highly conserved in 2,625
29 PPi-ehiAK homologs, implying that almost all organisms have ATP-dependent,
30 rather than PPi-dependent, AK.

31

32 **Key words**33 PPi; ATP; acetate kinase; *Entamoeba histolytica*; *Methanosarcina thermophila*

34

35

36 Introduction

37 Acetate kinase (AK) is a critical enzyme for central carbon metabolism in
38 bacteria and archaea (1). ATP-dependent AK catalyzes the phosphorylation of
39 acetate to produce acetyl phosphate by utilizing ATP as a phosphoryl donor and
40 also catalyzes the reverse reaction (2). This enzyme has been purified and
41 characterized from various archaea and bacteria, including *Methanosarcina*
42 *thermophila*, *Escherichia coli*, *Thermotoga maritima*, and *Lactobacillus*
43 *sanfranciscensis* (3-7). The three-dimensional structure of AK of *M. thermophila*
44 (mthAK) has been solved (8) and several key catalytic residues have been
45 identified based on the tertiary structural information and site-directed
46 mutagenesis (8-15).

47 Flower et al. have recently shown that the AK of the amitochondriate protist
48 *Entamoeba histolytica* (PPi-ehiAK) is a novel PPi-dependent AK that
49 phosphorylates acetate using PPi, not ATP, as a phosphoryl donor (16). The
50 three-dimensional structure of PPi-ehiAK has been solved and the
51 substrate-binding site of PPi-ehiAK has been compared with that of mthAK,
52 although without a biochemical verification (17). Despite the differences in
53 phosphoryl donor specificity, the primary sequences of AK enzymes are highly
54 conserved (8, 17).

55 The significance of AK in central carbon and energy metabolism indicates the
56 importance of understanding the determinants of the specificity of AK for
57 phosphoryl donors (ATP and PPi). Such understanding is also important in a
58 structural biology context, given the structural overlap between ATP and PPi (Fig.
59 1). In this study, we verified that PPi-ehiAK is specific for PPi, and does not

60 accept ATP; and that *E. coli* AK (ATP-ecoAK) is specific for ATP, and not for PPi.
61 We then identified the amino acid determinants of the specificity to ATP of
62 ATP-ecoAK based on a comparison of the tertiary structures of mthAK and
63 PPi-ehiAK and the primary structures of mthAK, PPi-ehiAK, and ATP-ecoAK,
64 combined with site-directed mutagenesis of ATP-ecoAK.

65

66 MATERIALS AND METHODS

67

68 **Expression of PPi-ehiAK** *PPi-ehiAK* (KEGG ID, ehi, EHI_170010) with
69 optimized codon usage for *E. coli* was synthesized at Operon (Tokyo, Japan)
70 (Supplementary Fig. S1). *PPi-ehiAK* was amplified by PCR using the synthesized
71 gene as a template with primers AK_pQE_B_F (5'-TCA CCA TCA CGG ATC
72 CAT GTC TAA TGT GCT GAT TTT C-3', with the *Bam*HI site underlined) and
73 AK_pQE_S_R (5'-GCT GCA GGT CGA CCC TTA AAA CTG GAA TAA TTC
74 TTT C-3'). The PCR product was inserted into *Bam*HI/*Sma*I sites in pQE-80L
75 with In-Fusion (Clontech, Otsu, Japan), yielding pMK3549. *E. coli* MK3648
76 strain was obtained by transforming *E. coli* NovaBlue (Novagen) with pMK3549
77 and plasmid pLysSRARE (Novagen). PPi-ehiAK is expressed as a N-terminally
78 His-tagged protein in *E. coli* MK3648.

79 For expression of PPi-ehiAK, a fresh single colony of MK3648 strain that had
80 just been transformed was inoculated into 10 mL LB medium supplemented with
81 100 µg/mL ampicillin, 34 µg/mL chloramphenicol, and 12.5 µg/mL tetracycline.
82 After culturing at 37°C aerobically overnight, the culture was transferred to the
83 same medium (250 mL) and cultured at 37°C aerobically overnight. This culture

84 was again transferred to the same medium (4.5 L, 500 mL in a 2.0 L Sakaguchi
85 flask) and cultivation was continued at 37°C aerobically until A_{600} reached 0.4.
86 Then, isopropyl-1- β -D-thiogalactopyranoside was added to 1 mM and cultivation
87 was continued further at 37°C aerobically for 1 h.

88

89 **Purification of PPi-ehiAK** MK3648 cells overexpressing PPi-ehiAK were
90 collected by centrifugation at 8,500 g for 10 min, suspended in 50 mL of 20 mM
91 HEPES-NaOH (pH 7.5), and disrupted by sonication at 4°C for 15 min with an
92 Insonator 201M (Kubota, Tokyo, Japan). After centrifugation at 20,000 g for 10
93 min, the clear supernatant was used as the cell extract containing PPi-ehiAK. This
94 cell extract was applied to a Talon Metal Affinity Resin column (2.5×4.5 cm)
95 (Clontech) equilibrated with 20 mM HEPES-NaOH (pH 7.5). After washing with
96 150 mL of 20 mM HEPES-NaOH (pH 7.5) containing 30 mM imidazole and 300
97 mM NaCl, PPi-ehiAK was eluted with 150 mL of 20 mM HEPES-NaOH (pH 7.5)
98 containing 150 mM imidazole and 300 mM NaCl. The fractions containing
99 PPi-ehiAK were combined, dialyzed against 20 mM HEPES-NaOH (pH 7.5) at
100 4°C overnight, and used as the purified PPi-ehiAK. If necessary, the purified
101 enzyme was concentrated by 3-fold with a Centriprep 10K instrument (Millipore).

102

103 **Expression of ATP-ecoA** For expression of ATP-ecoAK (KEGG ID, ecj,
104 Y75_p2262), *E. coli* K12 AG1 ASKA GFP-free strain (MK3814 strain), which
105 contained plasmid pMK3814 (pCA24N carrying the *ATP-ecoAK* gene without
106 GFP) (18) and was stored at -80°C in the presence of 17% v/v glycerol, was
107 inoculated into 20 mL LB medium supplemented with 34 μ g/mL chloramphenicol

108 and cultured at 37°C aerobically overnight. This culture was transferred to the
109 same medium (700 mL, 350 mL in a 500 mL Sakaguchi flask) and cultivation was
110 continued at 37°C aerobically until A_{600} reached 0.6. Then,
111 isopropyl-1- β -D-thiogalactopyranoside was added to 0.1 mM and cultivation was
112 continued at 37°C aerobically for 3 h. Purification and dialysis were conducted as
113 described for P*Pi*-ehiAK above, but with use of 10 mM Tris-HCl (pH 8.0) instead
114 of 20 mM HEPES-NaOH (pH 7.5).

115

116 **Expression of variant ATP-ecoAK** Site-directed mutagenesis was performed
117 using inverse PCR followed by *DpnI* treatment using pMK3814 as a template.
118 The variant ATP-ecoAK plasmids were confirmed by DNA sequencing. These
119 plasmids were introduced into the *E. coli* DH5 α strain and each variant
120 ATP-ecoAK was expressed and purified as described below for ATP-ecoAK.

121

122 **Assay of AK activity** The AK activity in the acetyl phosphate-forming
123 direction was assayed at 30°C using a hydroxamate assay that detects formation of
124 acetyl phosphate (2, 19). The assay utilizes the reaction of acetyl phosphate with
125 hydroxylamine to form acetyl hydroxamate, which forms a colored complex with
126 trivalent iron. The reaction mixture (333 μ L) comprised 10 mM phosphoryl donor,
127 150 mM HEPES-NaOH (pH 6.5), 200 mM potassium acetate, 10 mM MgCl₂, 700
128 mM hydroxylamine hydrochloride, with the pH adjusted to pH 6.5 with KOH just
129 before use, and an appropriate amount of AK. The reaction was initiated by
130 addition of AK and terminated by addition of 333 μ L of 10% trichloroacetic acid,
131 followed by addition of 333 μ L of 2.5% FeCl₃ in 2.0 N HCl. After incubation for

132 5 min, the absorbance at 540 nm (A_{540}) was measured and defined as $^1A_{540}$. PPi
133 (Nakalai Tesque, Kyoto, Japan) and ATP (Wako Pure Chemical) were used as
134 phosphoryl donors. AK was diluted with 20 mM HEPES-NaOH (pH 7.5) or 10
135 mM Tris-HCl (pH 8.0) if required. Control reactions were conducted without a
136 phosphoryl donor and the resultant A_{540} was defined as $^2A_{540}$. AK activity was
137 calculated as $\Delta A_{540} (^1A_{540} - ^2A_{540})$.

138 The AK activity in the PPi-forming direction was assayed at 30°C by detecting
139 formation of PPi using a PPiLight™ inorganic pyrophosphate assay (Lonza, Basel,
140 Switzerland) (20, 21). This assay utilizes the reactions of PPi with AMP to form
141 ATP, and of luciferase producing light from the newly formed ATP. The reaction
142 mixture (50 μ L) comprised 100 mM HEPES-NaOH (pH 6.5), 20 mM potassium
143 phosphate (Pi) (pH 7.0), 20 mM $MgCl_2$, 1.5 mM acetyl phosphate, and an
144 appropriate amount of AK. The reaction was initiated by addition of AK and
145 terminated by boiling for 5 min. After the reaction, 20 μ L of PPiLight™
146 Converting Reagent was added to the reaction mixture (40 μ L) with appropriate
147 dilution with distilled water (e.g., 50-fold dilution for the PPi-ehiAK reaction).
148 After incubation at room temperature for 30 min, 20 μ L of PPiLight™ Detection
149 Reagent was added and the mixture (60 μ L) was further incubated at room
150 temperature for 30 min. Luminescence of the mixture was then read with a 0.1 s
151 integration time and the relative luminescent unit (RLU) value was measured as
152 1RLU . Control reactions were conducted without AK and the resultant RLU was
153 defined as 2RLU . AK activity in the PPi-forming direction was calculated as
154 $\Delta RLU (^1RLU - ^2RLU)$ using an authentic PPi as a standard.

155 One unit (U) of enzyme activity was defined as 1 μ mol acetyl phosphate

156 produced in 1 min at 30°C and specific activity was expressed in U/mg protein.
157 Protein concentrations were determined by the method of Bradford (22) with
158 bovine serum albumin as a standard.

159

160 **Differential scanning fluorimetry (DSF)** Interaction between AK and
161 substrate (ATP or PPi) was assessed by DSF (23) using a MyiQ2 real-time PCR
162 instrument (Bio-Rad). The fluorescence of SYPRO Orange (Invitrogen) was
163 monitored using filters provided with the PCR instrument (excitation at 492 nm
164 and emission at 610 nm). The reaction mixture (20 μ l) comprised 5 μ g AK, 20
165 mM Tris-HCl (pH 7.5), 1000-fold diluted SYPRO Orange, and each substrate (1.0
166 mM ATP, 5.0 mM PPi). This mixture was subjected to a temperature increase
167 from 20 to 95°C by 0.5°C/cycle for a total of 141 cycles. The fluorescence profile
168 was obtained by plotting the relative fluorescence unit (RFU) value at each
169 temperature. The profile was analyzed using *iQ5* (Bio-Rad) and the midpoint of
170 the increase in the profile was defined as the melting temperature (T_m).

171

172 **Elution of AKs from an ATP-agarose column** Purified ATP-ecoAK in 10
173 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$ (500 μ L, 1.0 mg/mL) was
174 applied to an ATP-agarose column (100 μ L) (Sigma-Aldrich) equilibrated with 10
175 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$. The column was allowed to
176 stand for 1 h at 4°C for binding to take place. After incubation, the column was
177 washed with 2 mL of 10 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$. Next,
178 500 μ L of 50 mM ATP (pH 7.0) containing 10 mM $MgCl_2$ was added and the
179 column was incubated for 30 min at 4°C, followed by elution of ATP-ecoAK. In

the case of purified PPi-ehiAK in 20 mM HEPES-NaOH (pH 7.5) containing 10 mM MgCl₂ (500 μL, 1.0 mg/mL), the application, washing, and elution procedures were the same as those for ATP-ecoAK, but with use of 20 mM HEPES-NaOH (pH 7.5) instead of 10 mM Tris-HCl (pH 8.0).

SDS-PAGE SDS-PAGE was performed on 12.5% acrylamide gels.(24) Proteins in the gel were visualized with Coomassie brilliant blue R-250.

RESULTS

Purification and phosphoryl donor specificity of PPi-ehiAK and ATP-ecoAK An *PPi-ehiAK* gene with optimized codon usage for *E. coli* was synthesized (Fig. S1), cloned in pQE-80L, and expressed as a N-terminally His-tagged protein in *E. coli*. The purified PPi-ehiAK protein was 44 kDa on SDS-PAGE, in agreement with the calculated molecular mass (44 kDa; His-tag, 1 kDa + PPi-ehiAK, 43 kDa) (Fig. S2). Kinetic constants showed that the purified PPi-ehiAK utilized PPi, but not ATP, as a phosphoryl donor (Table 1). Native ATP-ecoAK purified from *E. coli* utilizes ATP (7), but the strict phosphoryl donor specificity of ATP-ecoAK is unknown. To examine this issue, a N-terminally His-tagged ATP-ecoAK was also expressed in *E. coli*. The purified ATP-ecoAK was shown to be specific for ATP, and not to use PPi as a phosphoryl donor (Table 1).

Candidates for determinants of the phosphoryl donor specificity of AK

204 To probe the structural determinants of the phosphoryl donor specificity of AK,
205 we inspected the substrate-binding site of the tertiary structure of mthAK
206 complexed with acetate and ADP-AlF₃ (9). Noted that although mthAK utilizes
207 ATP for the phosphorylation of acetate (5), if mthAK uses PPi or not has not been
208 experimentally confirmed. We found 12 residues located within 4.0 Å of ADP:
209 Leu-209, Gly-210, Asn-211, Asp-283, Phe-284, Arg-285, Ala-330, Gly-331,
210 Ile-332, Asn-335, Ser-336, and Arg-362. Among these residues, 5 (Asn-211,
211 Ala-330, Gly-331, Ile-332, and Asn-335 of mthAK) are conserved in ATP-ecoAK,
212 but not in PPi-ehiAK, based on a multiple sequence alignment (Fig. 2). This
213 suggests that these residues are candidates for determinants of the phosphoryl
214 donor specificity. Four of these 5 residues (Asn-211, Gly-331, Ile-332, and
215 Asn-335) form hydrogen bonds and van der Waals contacts to ADP (Table S1). Of
216 the other 7 residues among the original 12 in mthAK, two (Ser-336 and Arg-362)
217 are not conserved and 5 (Leu-209, Gly-210, Asp-283, Phe-284, and Arg-285) have
218 defined roles (8, 12) and are conserved in both ATP-ecoAK and PPi-ehiAK (Fig.
219 2).

220 Among the 5 candidate residues, Asn-211, Gly-331, and Ile-332 of mthAK
221 have been proposed to bind to the β-phosphate, α-phosphate, and adenine base of
222 ADP, respectively, although this has not been confirmed experimentally (Fig. 3)
223 (8). Ala-330 is located close to the β-phosphate and Asn-335 interacts with the
224 adenine base of ADP (Table S1), suggesting that Ala-330 and Asn-335 are key
225 residues in recognition of ATP in mthAK (Fig. 3). Superposition of the
226 substrate-binding site of the tertiary structure of mthAK complexed with acetate
227 and ADP-AlF₃ (9) and that of PPi-ehiAK (17) showed that the corresponding 5

228 residues in PPi-ehiAK would occlude the ATP-binding cleft of mthAK and thus
229 could reject binding of ATP to PPi-ehiAK (Fig. 3). Consistent with this
230 observation, Gln-323 and Met-324 of PPi-ehiAK have previously been proposed
231 to occlude the ATP-binding cleft (17).

232

233 **Kinetic analyses of variant ATP-ecoAKs** The candidate residues in
234 mthAK/ATP-ecoAK/PPi-ehiAK are Asn-211/Asn-213/Thr-201,
235 Ala-330/Gly-332/Asp-322, Gly-331/Gly-333/Gln-323, Ile-332/Ile-334/Met-324,
236 and Asn-335/Asn-337/Glu-327 (Figs. 2 and 3). To examine the roles of these
237 residues in phosphoryl donor specificity, each candidate residue in ATP-ecoAK,
238 which is specific for ATP (Table 1), was substituted with the respective PPi-ehiAK
239 residue and the variant ATP-ecoAKs were purified (Fig. S2). All 5 variant
240 ATP-ecoAKs exhibited increased K_m and decreased V_{max} for ATP (Table 1),
241 indicating that these residues are the determinants for the specificity to ATP in
242 ATP-ecoAK. Notably, K_m for ATP of ATP-ecoAK N337E increased 46-fold
243 relative to ATP-ecoAK, showing a critical role of ATP-ecoAK Asn-337 in ATP
244 binding. Thus, Glu-327 of PPi-ehiAK may be a particularly potent determinant of
245 rejection of ATP and promotion of PPi specificity in PPi-ehiAK (Fig. 3, Table 1).
246 Each variant ATP-ecoAK showed no PPi-dependent AK activity for both acetyl
247 phosphate and PPi formation (Tables 1 and S2).

248

249 **Analyses of substrate binding to AKs** Affinities of PPi-ehiAK, ATP-ecoAK,
250 and variant ATP-ecoAKs for each ATP and PPi were analyzed using DSF based on
251 changes in protein stability upon ligand binding (23). The fluorescence of SYPRO

Orange bound to denatured proteins was measured during heat treatment from 25 to 95°C. T_m values of AKs in the absence and presence of substrates were determined as the midpoint of the increase in the fluorescence profile (Table 2). The difference in T_m of native ATP-ecoAK in the presence and absence of ATP ($\Delta T_{m \text{ ATP}}$) was 13°C, suggesting that the thermal stability of ATP-ecoAK was increased by the binding of ATP. In contrast, $\Delta T_{m \text{ ATP}}$ of PPi-ehiAK could not be measured, concordant with the absence of ATP-dependent activity of PPi-ehiAK (Table 1). $\Delta T_{m \text{ ATP}}$ of all variant ATP-ecoAKs was decreased significantly relative to that of native ATP-ecoAK, in agreement with the decreased activity of variant ATP-ecoAK and supporting the roles of the 5 residues as determinants of specificity for ATP. The reason for the greatly decreased $\Delta T_{m \text{ ATP}}$ for ATP-ecoAK G332D remains to be clarified.

To confirm the ATP-binding data, ATP-ecoAK, ATP-ecoAK N337E, and PPi-ehiAK were added to an ATP-agarose column, washed, and eluted with 50 mM ATP containing 10 mM MgCl_2 . As expected, ATP-ecoAK was eluted by ATP after complete washing, while PPi-ehiAK was not eluted (Fig. S3), in agreement with the absence of ATP-dependent activity of PPi-ehiAK and the DSF results (Tables 1 and 2). Slight elution occurred for ATP-ecoAK N337E (Fig. S3), which had a significantly increased K_m for ATP (Table 1). This confirms that the affinity of ATP-ecoAK N337E for ATP was decreased by substitution of Asn-337 to Glu.

$\Delta T_{m \text{ PPi}}$ of PPi-ehiAK was 1.7, indicating the expected binding of PPi to PPi-ehiAK (Table 2). Unexpectedly, $\Delta T_{m \text{ PPi}}$ of ATP-ecoAK was 4.4, suggesting that PPi can bind to ATP-ecoAK, although ATP-ecoAK cannot utilize PPi as a phosphoryl donor for catalysis (Table 1). $\Delta T_{m \text{ PPi}}$ of ATP-ecoAK was not markedly

reduced upon substitution of each candidate residue, especially Gly-333, Ile-334, and Asn-337, compared to the effects on ΔT_m ATP (Table 2). This may reflect the role of these residues in binding to adenosine of ADP, and thus not to PPi (Fig. 3). Substitution of Asn-213 or Gly-332 of ATP-ecoAK reduced ΔT_m PPi of ATP-ecoAK. This is in agreement with the putative binding of Asn-213 of ATP-ecoAK to the β -phosphate of ADP and putative equivalent binding of Asn-211 of mthAK to the PPi moiety. Gly-322 of ATP-ecoAK is located close to the β -phosphate of ADP, and Ala-330 of mthAK may similarly be located close to PPi (Fig. 3).

Asn-213 and Gly-332 of ATP-ecoAK correspond to Thr-201 and Asp-322 of PPi-ehiAK respectively (Figs. 2 and 3). To examine the involvement of Thr-201 and Asp-322 of PPi-ehiAK in utilization of PPi, we focused on the residues (Leu-326 and Asn-376) of PPi-ehiAK that interact with Thr-201 and Asp-322 (Table S3, Fig. S4). Leu-326 and Asn-376 of PPi-ehiAK correspond to Glu-336 and Thr-385 of ATP-ecoAK. Glu-336, Thr-385, Asn-213 and Gly-332 of ATP-ecoAK were substituted with the respective PPi-ehiAK residues (Leu-326, Asn-376, Thr-201, and Asp-322) to give a quadruple ATP-ecoAK variant (ATP-ecoAK N213T G332D E336L T385N) that had the possibility of utilizing PPi (Fig. S2). However, this variant could not utilize PPi and ΔT_m PPi was not increased by these substitutions. V_{max} and k_{cat} for ATP were significantly decreased in the quadruple variant (Table 1) and ΔT_m ATP was markedly decreased (Table 2).

DISCUSSION

300 The results of the study show that PPi-ehiAK is specific for PPi and does not
301 utilize ATP (16), while ATP-ecoAK is specific for ATP and does not use PPi.
302 Based on the tertiary and primary structures and catalytic and binding activities of
303 variant enzymes, we identified 5 residues (Asn-213, Gly-332, Gly-333, Ile-334,
304 and Asn-337 in ATP-ecoAK) as determinants of specificity to ATP in ATP-ecoAK.

305 ATP-ecoAK N337E exhibited a 46-fold increase in K_m for ATP relative to
306 ATP-ecoAK, indicating that Asn-337 of ATP-ecoAK is the most crucial for the
307 specificity to ATP and suggesting that the corresponding Asn-335 in mthAK is
308 also crucial for binding to ATP and that the corresponding Glu-327 in PPi-ehiAK
309 is possibly important for rejection of ATP in PPi-ehiAK. The basis of the role of
310 Asn-337 of ATP-ecoAK in accepting ATP was examined using molecular and
311 electrostatic surfaces of the ADP-binding sites of mthAK N335E and mthAK, for
312 which a tertiary structure has been solved (9). The ADP-binding cleft is negatively
313 charged in mthAK N335E, but positively charged in mthAK (Fig. S5). There was
314 no clear structural change in the molecular surface of mthAK N335E that could
315 have resulted in rejection of ATP. Thus, the negatively charged ATP-binding site
316 in ATP-ecoAK N337E may explain the weaker affinity for ATP.

317 To get further insight into the substrate specificity of AKs, we examined how
318 the 5 key residues are distributed in the primary structures of AKs. A total of
319 2,625 proteins homologous to PPi-ehiAK from all species with sequenced
320 genomes were found using BLASTP (25). Among the homologs, the 5 key
321 residues of mthAK/ATP-ecoAK/PPi-ehiAK (Asn-211/Asn-213/Thr-201,
322 Ala-330/Gly-332/Asp-322, Gly-331/Gly-333/Gln-323, Ile-332/Ile-334/Met-324,
323 and Asn-335/Asn-337/Glu-327) are highly conserved as Asn, Ala or Gly, Gly, Ile,

324 and Asn, respectively (Table 3). These are mthAK- and ATP-ecoAK-specific
325 residues, which suggests that almost all organisms have ATP-dependent AKs. In
326 contrast, 10 PPi-ehiAK homologs, including PPi-ehiAK, possess the
327 PPi-ehiAK-specific residue (Glu) in the most crucial position determining the
328 phosphoryl donor specificity (corresponding to Asn-335/Asn-337/Glu-327 in
329 mthAK/ATP-ecoAK/PPi-ehiAK) (Table 3). However, except for PPi-ehiAK and
330 the PPi-ehiAK homolog from *Entamoeba dispar*, which is closely related to *E.*
331 *histolytica*, 8 of these homologs have no PPi-ehiAK-specific residues in the other
332 4 positions (corresponding to Thr-201, Asp-322, Gln-323, and Met-324 in
333 PPi-ehiAK) (Table S4). This suggests that only the PPi-ehiAK homolog from *E.*
334 *dispar* is PPi-dependent. The phosphoryl donor specificities of the other 8
335 PPi-ehiAK homologs remain to be clarified.

336

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341

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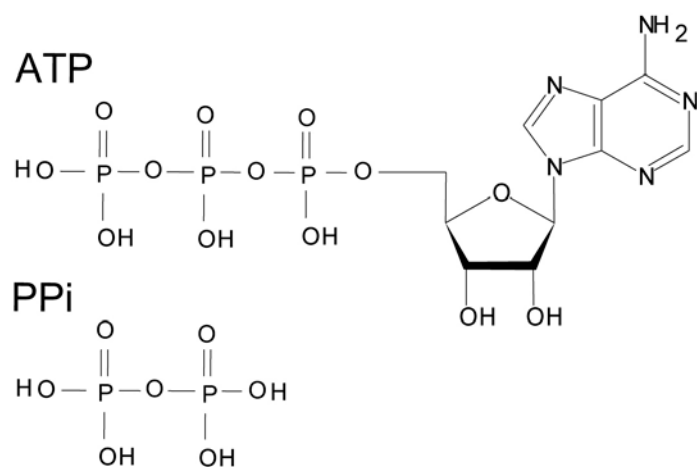
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- 430

431 **FIGURES**

432



433

434 **Fig. 1.** Structures of ATP and PPi.

435

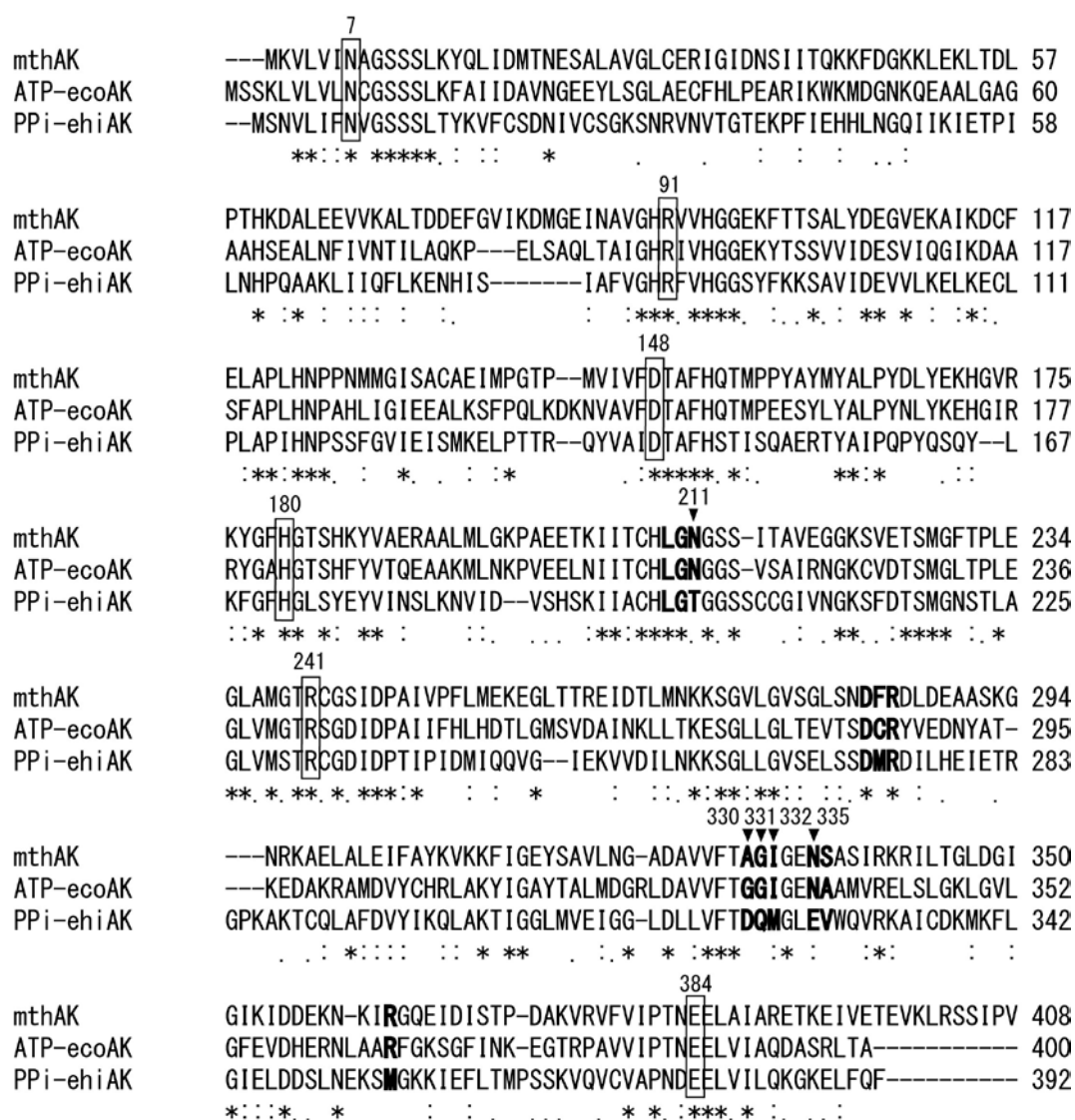


Fig. 2. Multiple alignment of the primary sequences of mthAK, ATP-ecoAK, and PPI-ehiAK. Multiple alignment of the mthAK (Uniprot, P38502), ATP-ecoAK (KEGG, Y75_p2262), and PPI-ehiAK (KEGG, EHI_170010) sequences was conducted using ClustalW2 (26). The 12 residues located within 4.0 Å of ADP in the tertiary structure of mthAK complexed with acetate and ADP-AlF₃ (9) are shown in bold. Among these 12 residues, the 5 candidate residues for determination of phosphoryl donor specificity are denoted by arrowheads. Key residues involved in catalysis in mthAK (Asn-7, Arg-91, Asp-148, His-180, Arg-241, and Glu-384) (8-10) are conserved in ATP-ecoAK and PPI-ehiAK and

are boxed. Identical residues are denoted by an asterisk (*), strongly conserved residues by a colon (:), and weakly conserved residues by a period (.). Numbers above the sequences refer to mthAK and numbering for each protein is shown on the right.

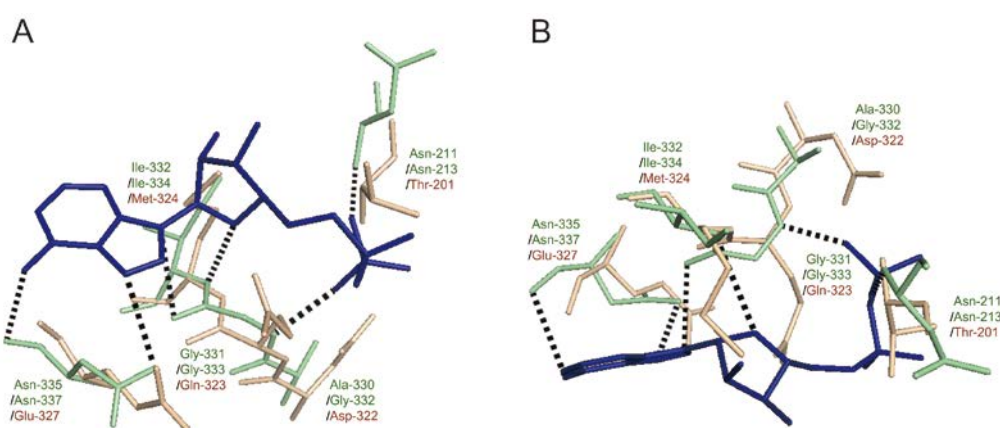


Fig. 3. Superposition of the substrate-binding site structures of mthAK and PPi-ehiAK. A, Superposition of the substrate-binding sites of PPi-ehiAK (PDB, 4H0O) (17) and mthAK complexed with acetate and ADP-AlF₃ (PDB, 1TU9) (9). Amino acid residues of mthAK are shown in green, ADP in mthAK is in deep blue, and PPi-ehiAK is in light orange. Residue names and numbers are shown as mthAK (green)/ATP-ecoAK (green)/PPi-ehiAK (pink). Hydrogen bonds in mthAK are shown as dashed lines. B, Overhead view of A. Superposition of AKs was performed using PyMOL.

Table 1. Kinetic constants of AKs.^a

Phosphoryl donor	PPi			ATP		
	K_m	V_{max}	k_{cat}	K_m	V_{max}	k_{cat}
	(mM)	(U/mg)	(s ⁻¹)	(mM)	(U/mg)	(s ⁻¹)
PPi-ehiAK	3.3 ± 0.5	3.7 ± 0.2	2.6 ± 0.1	nd	nd	nd
ATP-ecoAK	nd ^b	nd	nd	0.67 ± 0.06	1258 ± 28	908 ± 20
ATP-ecoAK N213T	nd	nd	nd	1.7 ± 0.2	22.7 ± 1.1	16.3 ± 0.8
ATP-ecoAK G332D	nd	nd	nd	3.5 ± 0.4	13.7 ± 0.7	9.9 ± 0.5
ATP-ecoAK G333Q	nd	nd	nd	5.2 ± 0.4	56.8 ± 1.5	41.0 ± 1.1
ATP-ecoAK I334M	nd	nd	nd	6.6 ± 0.6	72.8 ± 3.3	10.5 ± 0.5
ATP-ecoAK N337E	nd	nd	nd	30.7 ± 1.9	537 ± 24	395 ± 18
ATP-ecoAK N213T G332D E336L T385N	nd	nd	nd	0.66 ± 0.18	0.47 ± 0.01	0.34 ± 0.01
PPi-ehiAK ^c	3.6 ± 0.1	nr	nr	nr	nr	nr
ATP-ecoAK ^d	nr	nr	nr	0.07	2,000	nr

^a AK activity was assayed with different concentrations of PPi or ATP. nd, not detected. nr, not reported. Kinetic values were calculated by fitting data to the appropriate Michaelis-Menten equations using KaleidaGraph software (Synergy Software). ^b ΔA_{540} was 0.11 in the presence of 5 mM PPi with 15.8 μ g of PPi-ehiAK after a 10 min reaction, but ΔA_{540} was zero in the presence of 10 mM PPi with 20 μ g of ATP-ecoAK, ATP-ecoAK N213T, ATP-ecoAK G332D, ATP-ecoAK G333Q, ATP-ecoAK I334M and ATP-ecoAK N337E after 10, 30 and

60 min; thus AK activities of ATP-ecoAK and variant ATP-ecoAK toward PPi were regarded as not detected (nd). ΔA_{540} was 0.036 in the presence of 10 mM ATP with 6.4×10^{-3} μ g of ATP-ecoAK after a 10 min reaction, but ΔA_{540} was zero in the presence of 5 mM ATP with 15.8 μ g of PPi-ehiAK after 10, 30 and 60 min; thus AK activities of PPi-ehiAK toward ATP were regarded as not detected. ^c Data from a previous report (16). ^d Data from a previous report (7).

Table 2. Differences in T_m of AKs in the absence and presence of ATP and PPi.

Enzyme	$\Delta T_{m \text{ ATP}} (^{\circ}\text{C})$	$\Delta T_{m \text{ PPi}} (^{\circ}\text{C})$
PPi-ehiAK	nd	1.7 \pm 0.2
ATP-ecoAK	12.5 \pm 0.9	4.4 \pm 0.6
ATP-ecoAK N213T	1.9 \pm 0.1	1.7 \pm 0.6
ATP-ecoAK G332D	0.22 \pm 0.18	2.0 \pm 0.1
ATP-ecoAK G333Q	2.7 \pm 0.1	3.7 \pm 0.2
ATP-ecoAK I334M	1.3 \pm 0.2	3.3 \pm 0.2
ATP-ecoAK N337E	1.0 \pm 0.1	3.4 \pm 0.3
ATP-ecoAK N213T G332D E338L T385N	0.73 \pm 0.20	2.7 \pm 0.2

Differences in T_m of AKs in the absence and presence of ATP and PPi ($\Delta T_{m \text{ ATP}}$ and $\Delta T_{m \text{ PPi}}$) are shown. ATP was used at 1.0 mM and PPi at 5.0 mM. Means and standard deviations of three independent experiments are shown. nd, not detected.

Table 3. Distribution of the 5 candidate residues in AK sequences in a database search.^a

Candidate residues of mthAK/ATP-ecoAK/PPi-ehiAK	of mthAK/ATP-ecoAK-specific residues	PPi-ehiAK-specific residues
Asn-211/Asn-213/Thr-201	Asn (2,100/80%) ^b	Thr (2/0.08%)
Ala-330/Gly-332/Asp-322	Ala or Gly (2,584/98%)	Asp (3/0.11%)
Gly-331/Gly-333/Gln-323	Gly (2,581/98%)	Gln (2/0.08%)
Ile-332/Ile-334/Met-324	Ile (1,752/67%)	Met (78/3%)
Asn-335/Asn-337/Glu-327	Asn (2,101/80%)	Glu (10/0.38%)

^a Distribution of the 5 candidate residues of mthAK/ATP-ecoAK/PPi-ehiAK (Asn-211/Asn-213/Thr-201, Ala-330/Gly-332/Asp-322, Gly-331/Gly-333/Gln-323, Ile-332/Ile-334/Met-324, and Asn-335/Asn-337/Glu-327) in a database search of AKs. A total of 2,625 PPi-ehiAK homologs were found using BLASTP (25) on the GenomeNet server (<http://www.genome.jp>). ^b Number and percentage (%) of PPi-ehiAK homologs possessing the indicated amino acid at the position of the candidate residue.

Supplementary information

Table S1. Interactions between mthAK and ADP ^a

Hydrogen bonds				van der Waals contacts		
ADP atoms	Target atoms	Distance (Å)	ADP atoms	Target atoms	Distance (Å)	
β-phosphate						
O2B	Asn-211 ^b N	2.7				
O3B	Gly-331 ^b N	2.6				
Ribose						
O2'	Phe-284 ^c N	3.3	C1'	Gly-331 ^b C	3.7	
O3'	Asp-283 ^c OD2	3.3		Ile-332 ^b CG1	3.5	
O4'	Ile-332 ^b N	3.6	C2'	Phe-284 ^c CB	3.9	
Adenine						
N3	Arg-285 ^c N	3.5	C2	Arg-285 ^c CA	3.6	
N6	Asn-335 ^b O	3.4		CB	3.9	
	Arg-362 ^d NH ₂	3.2		Ser-336 ^d CB	3.8	
N7	Asn-335 ^b ND2	3.2	C5	Asn-335 ^b CB	3.8	
N9	Gly-331 ^b O	3.4				

^a Interactions between ADP and residues of mthAK B chain. ^b Five candidate residues specific to PPi-ehiAK. ^c Five residues conserved in mthAK, ATP-ecoAK, and PPi-ehiAK.

^d Residues not conserved.

Table S2. AK activity in the PPi-forming direction using Pi as a phosphoryl acceptor ^a.

Enzyme	PPi (μM)
PPi-ehiAK	3.1
ATP-ecoAK	nd ^b
ATP-ecoAK N213T	nd
ATP-ecoAK G332D	nd
ATP-ecoAK G333Q	nd
ATP-ecoAK I334M	nd
ATP-ecoAK N337E	nd

^a AK activity in the PPi-forming direction was assayed as described in the Materials and Methods. After reaction for 20 min with 1.4×10^{-2} μg of PPi-ehiAK, the reaction mixture was diluted 50-fold with distilled water and RLU was measured. PPi at 1.0 μM gave ΔRLU of 955,937. nd, not detected.

^b ΔRLU was zero with 9.2, 3.1, 2.4, 5.8, 2.9, and 3.1 μg of ATP-ecoAK, ATP-ecoAK N213T, ATP-ecoAK G332D, ATP-ecoAK G333Q, ATP-ecoAK I334M and ATP-ecoAK N337E, respectively, after the reaction for 20 min; thus, AK activities in the PPi-forming direction of ATP-ecoAK and these mutated ATP-ecoAKs using PPi were regarded as not detected (nd).

Table S3. Residues interacting with Thr-201 and Asp-322 in PPI-ehiAK^a.

Hydrogen bonds				van der Waals contacts			
Target atoms		Distance (Å)		Target atoms		Distance (Å)	
Thr-201							
O	Gly-202 ^b	N	3.4				
Asp-322							
O	Leu-326 ^c	N	3.4				
OD1	His-198 ^b	ND1	3.3	CA	Asn-376 ^c	CG	3.9
		NE2	2.3	CB	Leu-199 ^b	C	3.4
OD2	Leu-199 ^b	O	3.6		Gly-200 ^b	CA	3.9
	Gly-200 ^b	N	3.3	CG	Leu-199 ^b	C	3.9
N	Asn-376 ^c	OD1	3.1		Gly-200 ^b	CA	3.4

^a The residues interacting with Thr-201 and Asp-322 in PPI-ehiAK. Thr-201 and Asp-322 are expected to be around the phosphate group. ^b Four residues conserved in the primary structures of mthAK, ATP-ecoAK, and PPI-ehiAK. ^c Two residues conserved in ATP-dependent AKs, but not in PPI-ehiAK. This is the reason why we focused on these residues (Leu-326 and Asn-376 in PPI-ehiAK).

Table S4. Distribution of the 5 candidate residues.

Homolog name	Species name	Amino-acid residue corresponding to				
		Glu-327	Thr-201	Asp-322	Gln-323	Met-324
		of PPI-ehiAK				
EDI_237080 ^a	<i>Entamoeba</i> <i>dispar</i>	Glu	Thr	Asp	Gln	Met
GM21_0328 ^a	<i>Geobacter</i> sp. M21	Glu	Asn	Tyr	Gly	Glu
Gbem_0348 ^a	<i>Geobacter</i> <i>bemidjiensis</i>	Glu	Asn	Tyr	Gly	Glu
GM18_0425 ^a	<i>Geobacter</i> sp. M18	Glu	Asn	Tyr	Gly	Glu
Tpet_1041 ^a	<i>Thermotoga</i> <i>petrophila</i>	Glu	Ser	Gly	Gly	Met
Tnap_1061 ^a	<i>Thermotoga</i> <i>naphthophila</i>	Glu	Ser	Gly	Gly	Met
TRQ2_1071 ^a	<i>Thermotoga</i> sp. RQ2	Glu	Ser	Gly	Gly	Met
Tmari_1762 ^a	<i>Thermotoga</i> <i>maritima</i>	Glu	Ser	Gly	Gly	Met
TM1754 ^a	<i>Thermotoga</i> <i>maritima</i>	Glu	Ser	Gly	Gly	Met
ATP-ecoAK ^{a, b}	<i>E. coli</i>	Asn	Asn	Gly	Gly	Ile
mthAK	<i>M. thermophila</i>	Asn	Asn	Ala	Gly	Ile

^a Proteins in KEGG database (<http://www.genome.jp/kegg/>).

^b Y75_p2262 in KEGG database.

CGCGCGGCAGCCCATATGTCTAATGTGCTGATTTTCAACGTAGGATCTAGTTCCTCACATATAAA
GTGTTTTGTAGCGACAACATCGTCTGTAGTGGCAAATCCAATCGCGTGAATGTGACTGGTACTG
AGAAACCGTTTATCGAACATCACTTAAACGGCCAAATCATTAAAGATAGAAACCCCAATCCTCAA
CCATCCACAGGCTGCAAAACTGATTATCCAGTTCCTGAAAGAGAACCACATCTCTATTGCCTTT
GTAGGCCATCGCTTTGTTCATGGCGGGTCTTACTTTAAGAAATCAGCCGTCATTGACGAAGTCG
TGCTGAAAGAACTGAAAGAATGCCTGCCTTTAGCGCCCATTCATAACCCGAGCTCTTTCGGAG
TTATCGAAATCTCGATGAAAGAACTGCCAACCCTCGTCAGTATGTGGCGATCGATACGGCGTT
CCATTCGACCATTTCCCAAGCTGAACGCACCTATGCGATTCCGCAGCCGTATCAATCGCAGTAT
CTGAAATTCGGCTTTCACGGCCTGTCATACGAATACGTGATCAACTCGTTGAAGAATGTCATTG
ACGTAAGTCACAGCAAAATCATTGCATGCCATCTTGGTACAGGTGGCTCCTCATGCTGCGGCAT
TGTGAATGGGAAATCGTTTGATACGAGTATGGGTAACAGCACACTTGCTGGTCTGGTGATGTCA
ACGCGTTGTGGGGATATTGATCCCACCATTCCGATTGATATGATCCAGCAAGTGGGTATTGAGA
AAGTTGTCGATATTCTCAACAAGAAAAGCGGCTTGCTTGGGGTCAGTGAAGTGAAGCAGCGATA
TGCGCGATATTTTGACGAAATCGAAACCCGTGGTCCTAAAGCCAAAACGTGCCAACTGGCCT
TTGATGTTTACATCAAACAGCTGGCGAAAACCATCGGAGGCCTGATGGTTGAGATCGGCGGTC
TTGACTTACTGGTGTTACCGATCAGATGGGTCTGGAAGTATGGCAAGTTCGGAAAGCGATATG
CGACAAAATGAAATTTCTGGGCATTGAGTTGGACGATAGCCTCAATGAGAAGTCCATGGGTAA
GAAAATTGAATTCCTGACGATGCCGAGCAGCAAGGTACAGGTTTGTGTTGCACCGAATGACGA
GGAATTAGTCATACTGCAGAAAGGGAAAGAATTATTCCAGTTTT**TAA**CTCGAGCACCACCACC

Fig. S1. The sequence of *PPi-ehiAK* with optimized codon usage for *E. coli*. The optimized *PPi-ehiAK* gene (KEGG ID; ehi: EHI_170010) was synthesized at Operon. The *Nde*I site (CATATG) and *Xho*I site (CTCGAG) are underlined. The start codon (ATG) and stop codon (TAA) are shown in bold.

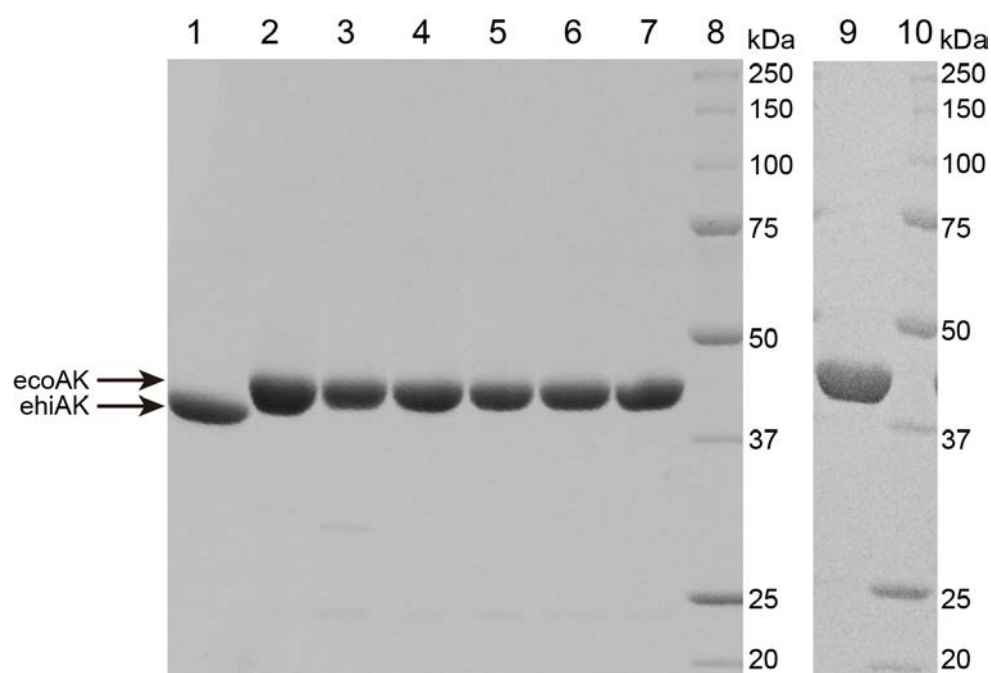


Fig. S2. SDS-PAGE of purified AKs. Lanes 1–7, 9: purified PPi-ehiAK, ATP-ecoAK, ATP-ecoAK N213T, ATP-ecoAK G332D, ATP-ecoAK G333Q, ATP-ecoAK I334M, ATP-ecoAK N337E, and ATP-ecoAK N213T G332D E336L T385N (5.0 μ g). Arrows indicate positions of purified AKs. Lanes 8 and 10: protein markers (Bio-Rad Laboratories, Hercules, CA).

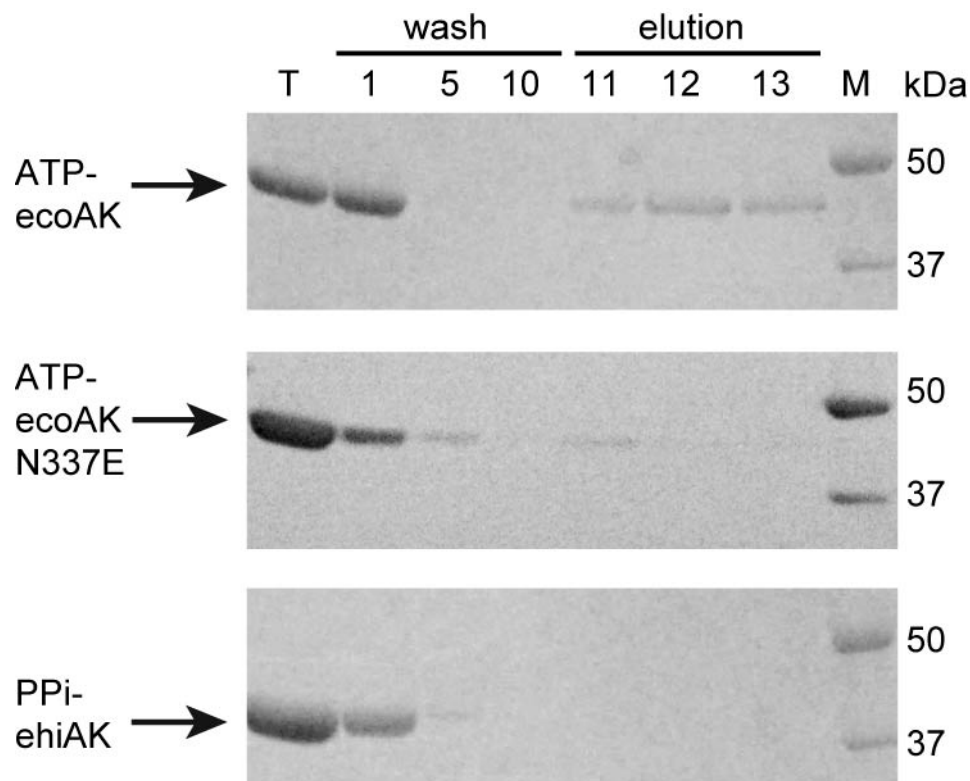


Fig. S3. SDS-PAGE of AKs eluted from an ATP-agarose column. Lane T: flow-through fraction; Lanes 1, 5, and 10: Fractions washed with 10 mM Tris-HCl (pH 8.0) containing 10 mM MgCl_2 or 20 mM HEPES-NaOH (pH 7.5) containing 10 mM MgCl_2 ; Lanes 11, 12, and 13: Fractions eluted with 50 mM ATP containing 10 mM MgCl_2 ; Lane M: protein markers. Lane numbers correspond to the numbers of fractions (200 μL each). Arrows indicate the positions of AKs.

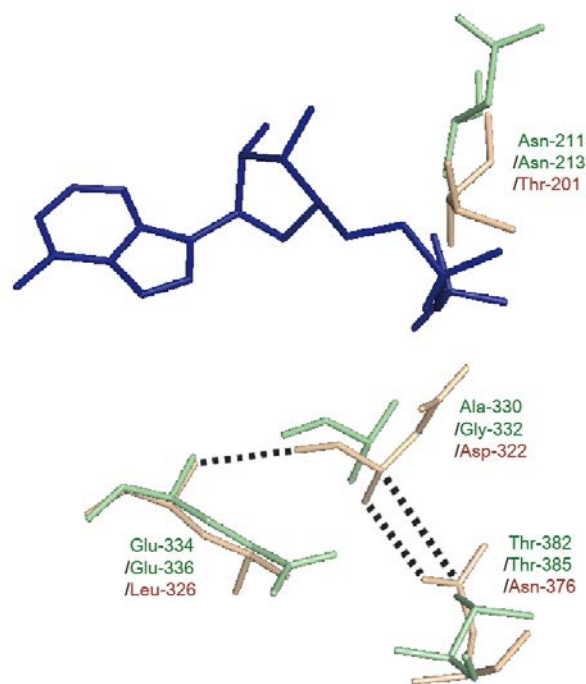


Fig. S4. Superposition of mthAK and PPi-ehiAK around the phosphate group of ADP. Superposition of the tertiary structure of PPi-ehiAK (PDB, 4H0O) (1) and that of mthAK complexed with acetate and ADP- AlF_3 (PDB, 1TU5) (2). Amino acid residues of mthAK are shown in green; ADP in mthAK is in deep blue, and PPi-ehiAK is in light orange. The residue names and numbers are shown for mthAK (green)/ATP-ecoAK (green)/PPi-ehiAK (pink). Hydrogen bonds in PPi-ehiAK are shown as dashed lines. Superposition of AKs was performed using PyMOL.

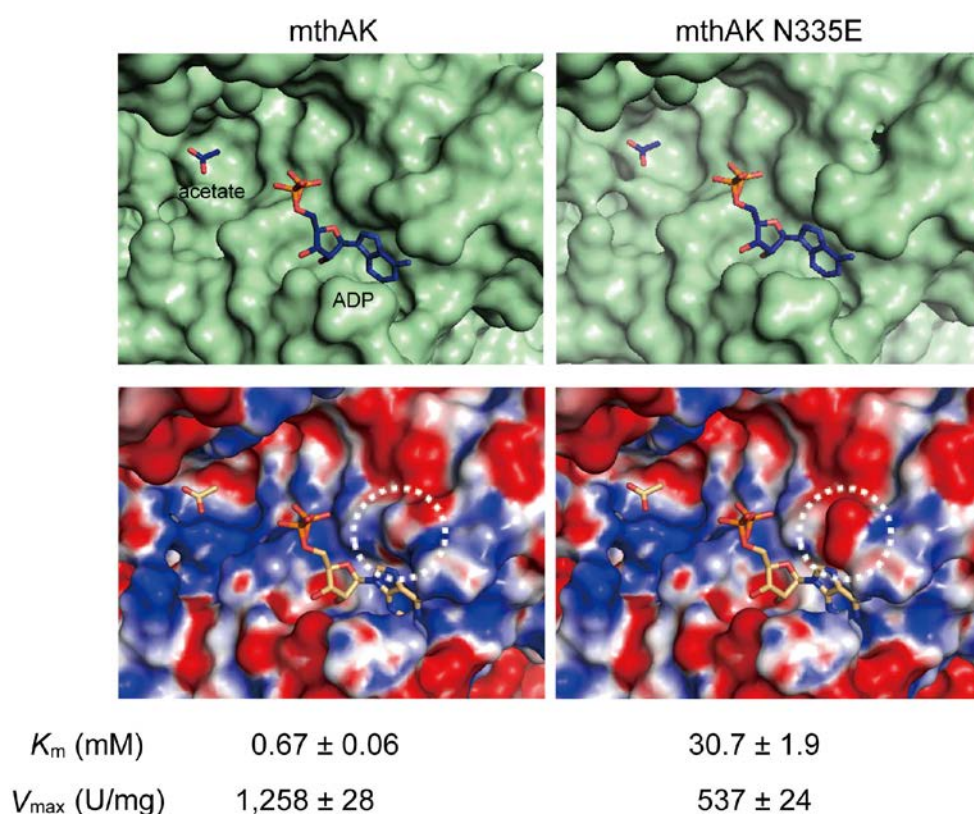


Fig. S5. ADP-binding sites of mthAK and mthAK N335E. Molecular surfaces of ADP-binding sites of mthAK (left) and mthAK N335E (right) are shown in the upper panels. Electrostatic surfaces of the AKs are shown in the lower panels. Positive and negative charges at pH 7.0 are colored in blue and red, respectively, and were calculated using APBS (3). The position around Asn-335 is denoted by a white dotted circle. K_m and V_{max} for ATP of ATP-ecoAK and ATP-ecoAK N337E are shown under the respective figures.

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